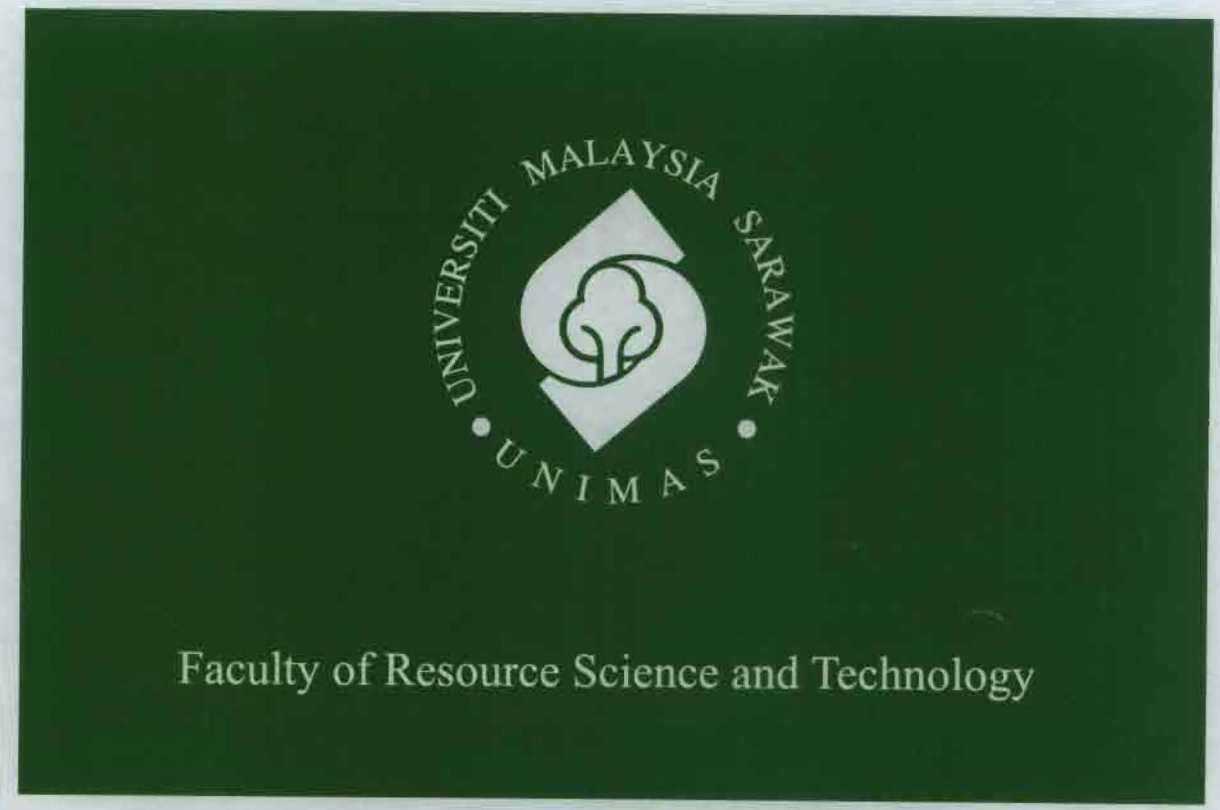

ISOLATION OF ANTIBIOTIC RESISTANCE AMONG *PSEUDOMONAS AERUGINOSA* FROM AQUACULTURE ENVIRONMENT AND RIVERS IN SARAWAK

Soh Khar Mun

Bachelor of Science with Honours
(Resource Biotechnology)
2015

Isolation of Antibiotic Resistance among *Pseudomonas aeruginosa* from Aquaculture Environment and Rivers in Sarawak

Soh Khar Mun (38898)

This project is submitted in fulfillment of the requirement for the Degree of Bachelor of Science with honors
(Resource Biotechnology)

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List of Abbreviation

µg	Microgram
µm	Micrometer
AmpC	Ampicillin class C
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CFU	Colony Forming Unit
ClO_3^-	Chlorate
CLSI	Clinical and Laboratory Standards Institute
CV-I	Crystal-violet-iodine
ddH_2O	Sterile distilled water
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EPS	Exopolysaccharide
EtBr	Ethidium bromide
ETC	Electron Transport Chain
FeS	Iron (II) sulphate
GDP	Gross Domestic Product
H_2O_2	Hydrogen peroxide
H_2S	Hydrogen sulphide
ICUs	Intensive Care Units
KIA	Klingler's Iron Agar
LB	Luria-Bertani Broth

mA	Milli ampere
$MgCl_2$	Magnesium chloride
mM	Milli mole
N_2	Nitrogen gas
N_2O	Nitrous oxide
NB	Nutrient Broth
NCBI	National Center for Biotechnology Information
NH_3	Ammonia
NO	Nitric oxide
NO_2	Nitrite
NO_3	Nitrate
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase Chain Reaction
PIA	*Pseudomonas* Isolation Agar
PNP	p-Nitrophenol
rpm	Revolutions per minute
spp.	Species
TBE	Tris-borate-EDTA
TNTC	Too numerous to count
TSA	Tryptic Soy Agar
TSI	Triple Sugar Iron
UV	Ultraviolet
V	Volt
w/v	Weight per volume

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Isolation of antibiotic resistance among *Pseudomonas aeruginosa* from aquaculture environment and river in Sarawak

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ABSTRACT

Pseudomonas aeruginosa, a Gram-negative bacillus-shaped bacterium can be found in various environments. The high adaptability of *P. aeruginosa* is devoted by the simple nutritional requirement and thus contributed *P. aeruginosa* as an opportunistic pathogen. In this study, *P. aeruginosa* was isolated from water and fish in rivers and aquaculture farm in Sarawak. A total of thirty-eight isolates were successfully isolated using CHROMagar™ *Pseudomonas*. The isolates were subjected to a series of biochemical tests such as gram staining, MacConkey agar test, oxidase test, triple sugar iron agar test, citrate test, catalase test, starch hydrolysis test, and nitrate reduction test. Based on the biochemical tests, two isolates possess the characteristics of *P. aeruginosa*. These isolates were further confirmed through molecular identification using 16S rRNA PCR analysis and DNA sequencing. These two *P. aeruginosa* were screened against nine antibiotics: piperacillin, ceftazidime, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, and norfloxacin using the disk diffusion assay. Both isolates were susceptible to all the antimicrobial agents tested. This suggested that all these antimicrobial agents are effective against *P. aeruginosa*.

Key words: *Pseudomonas aeruginosa*, aquaculture, biochemical test, 16S rRNA gene sequencing, antibiotic resistance

ABSTRAK

Pseudomonas aeruginosa, sejenis Gram-negatif bakteria berbentuk basillus boleh didapati dalam pelbagai persekitaran. P. aeruginosa mempunyai penyesuaian yang tinggi disebabkan keperluan khasiat yang mudah dan ini telah menyebabkan P. aeruginosa sebagai patogen oportunis. Dalam kajian ini, P. aeruginosa telah diambil dari air dan ikan di sungai dan ladang akuakultur di Sarawak. Sebanyak tiga puluh lapan isolat berjaya diasingkan dengan menggunakan CHROMagar™ Pseudomonas. Isolat ini diuji dengan satu siri ujian biokimia seperti pewarnaan gram, ujian agar MacConkey, ujian oxidase, ujian agar tiga gula besi, ujian sitrat, ujian katalase, ujian hidrolisis kanji hidrolisis, dan ujian pengurangan nitrat. Berdasarkan ujian biokimia, dua isolat yang memenuhi ciri-ciri P. aeruginosa. Isolat ini seterusnya dikenal pasti melalui pengenalan molekul dengan menggunakan analisis 16S rRNA PCR penjujukan DNA. Kedua-dua P. aeruginosa ini telah diskrinkan terhadap Sembilan antibiotic: piperacillin, ceftazidime, imipenem, meropenem, gentamicin, tobramycin, amikacin, ciprofloxacin, dan norfloxacin denganmenggunakan kaedah penyerapan cakera. Kedua-dua isolat terdedah kepada semua ejen antimikrob yang diuji. Ini mencadangkan bahawa semua ejen antimikrob berkesan untuk menentang P. aeruginosa.

Kata kunci: *Pseudomonas aeruginosa, akuakultur, ujian biokimia, penjujukan gen 16S rRNA, rintangan antibiotik*

1.0 Introduction

Aquaculture industry started in Malaysia since 1920 and today, this sector is one of the most important activities that contributed international trade, foreign exchange, and a significant source of animal protein (Hashim, 2008). In order to reach Malaysia's vision to become a high income country by 2020, the development of aquaculture into large-scale commercial industry is necessary. The increase demand for fish products especially in rural areas and the failure of captured fisheries to produce necessary fish products make aquaculture a major focus industry (Ling *et al.*, 2011). Therefore, the quality control of the production of fishery products is essential as consumption of fishery products which are raw or contaminated may poses health risks to the consumers.

Sarawak River, an important river basin in the state consists of two major tributaries. According to Nyambar and Bong (2010), these two principal tributaries are Sarawak River Kiri and Sarawak River Kanan. The total length of Sarawak River is 120 km with a total area of 2,456.04 km^2. Kuok *et al.* (2011) reported that Sarawak River covers towns like Bau, Batu Kawa, and Siniawan. In Sarawak, the establishment of aquaculture sector into commercial scale is markedly feasible due to the availability of water and land (Ling *et al.*, 2011).

Pseudomonas aeruginosa is one of the fish pathogens and can be found in soil and water. *P. aeruginosa*, a Gram-negative and non-fastidious bacterium with bacillus-shaped is highly adaptable in various environments. The bacterium requires only simple nutrient requirements and hence is an opportunistic pathogen that can cause infections in human, plant, and animals (Czekajlo-Kolodziej *et al.*, 2006; Franzetti & Scarpellini, 2007; Hirakawa *et al.*, 2010). Apart from that, the ability of *P. aeruginosa* as a multidrug resistant (MDR) and extremely drug resistant (XDR) made the treatment of disease a

difficult task since the bacterium capable to develop resistance during treatment course in a rapid manner (Lister *et al.*, 2009).

In this study, *P. aeruginosa* were isolated from aquaculture farm located in Sampadi, Bako, and Kampung Haji Baki. Besides that, cultured catfish from Kampung Haji Baki was also caught to isolate *P. aeruginosa*. In addition, water samples were collected from rivers located in Permai, Sarawak River Kiri (Muara Tuang and Bintawa), and Sarawak River Kanan (Siniawan and Waterfront). The detection of *P. aeruginosa* was performed using CHROMagar™ *Pseudomonas* as *Pseudomonas* spp. appears as blue colonies on the selective agar. Following that, the bacteria were characterized using conventional biochemical tests and identified through 16S rRNA gene sequencing. Antibiotic susceptibility test was then conducted and the pattern of antibiotic resistance pattern was analyzed.

The objectives of this study were:

1. to isolate *P. aeruginosa* from aquaculture environment and rivers
2. to determine the biochemical characteristics of isolated *P. aeruginosa* through bio-typing
3. to identify *P. aeruginosa* strains using 16S rRNA gene sequencing
4. to determine the antibiotic resistance pattern of *P. aeruginosa*

2.0 Literature Review

2.1 Rivers

Sarawak River consists of two major tributaries, is an important river basin in the state. The two principal tributaries are Sarawak River Kiri and Sarawak River Kanan. The total length of Sarawak River is 120 km which covers a total area of 2,456.04 km^2 (Nyambar & Bong, 2010). According to Kuok *et al.* (2011), Sarawak River is originated from Kapuas Mountains and discharged into South China Sea after collecting stormwater and wastewater from drains and tributaries. Sarawak River plays major role to serve residents in Kuching City for domestic and industrial use, to support tourism sector in the state, and function as tool to mitigate flood (Kuok *et al.*, 2011). Therefore, the rivers need to be preserved and managed properly.

2.2 Aquaculture Industry

In Malaysia, aquaculture industry started since 1920 from polyculturing Chinese carps in ex-mining pools. Today, vast variety of species such as marine shrimps, freshwater species, shellfishes, and marine finfishes are cultured using brackish water aquaculture, freshwater pond aquaculture, and marine aquaculture (Hashim, 2008). Aquaculture sector is now one of the most important activities which contributed 1.73% to Gross Domestic Product (GDP) in the country. Other than contributing to international trade and foreign exchange, aquaculture industry is also the cheapest source of animal protein especially for the rural communities (Hashim, 2008).

2.3 *Pseudomonas*

2.3.1 Phylogeny and Taxonomy

The genus *Pseudomonas* was first discovered by Carle Gessard from bluish-green pus in 1882 and this genus received names due to its blue-green coloration during culture (Lister *et al.*, 2009). However, the genus was described further in a vague manner in 1894. The name *Pseudomonas* created by Migula came from the combination of Greek and Latin words, pseudo- (false, Greek) and monas (single unit, Latin). Thus, term *Pseudomonas* means false single unit (Siegrist, 2010). *Pseudomonas* is one of the members of Gamma Proteobacteria class of bacteria and belongs to the Pseudomonadaceae family (Moore *et al.*, 2006).

2.3.2 Morphology

Pseudomonas is a Gram-negative bacterium with slightly curved or straight bacillus-shaped (Koneman, 1997; Czekajlo-Kolodziej *et al.*, 2006; Moore *et al.*, 2006; Franzetti & Scarpellini, 2007) measured from 0.5 to 1.0 μm in diameter by 1.5 to 5.0 μm in length (Moore *et al.*, 2006). Moore *et al.* (2006) stated that not all *Pseudomonas* will follow the traditionally described shape. *Pseudomonas putida* is extremely short while others can be unusually long. The motility of *Pseudomonas* is due to the presence of single polar flagellum and this enables *Pseudomonas* to react to chemical stimuli and localize low concentration organic substrates (Moore *et al.*, 2006).

2.3.3 Habitat

Pseudomonas species is highly adaptable in variety environments. Soil, water, plants and animals are common natural habitats of *Pseudomonas* spp. (Haynes, 1951; Franzetti & Scarpellini, 2007). An environment with a temperature range of 4 to 42 °C, pH from 4 to 8 and rich in organic compounds can likely be a habitat for *Pseudomonas* spp. Other than that, the supply of nitrate (NO_3) as the final electron acceptor enables *Pseudomonas* to survive in anaerobic condition (Moore *et al.*, 2006).

2.3.4 Physiology

The nutritional requirements of *Pseudomonas* spp. are simple (Franzetti & Scarpellini, 2007), thus the bacterium is also known as non-fastidious organism. Although the metabolism is carried out through respiration, some *Pseudomonas* spp. will carry out arginine fermentation as well as pyruvate fermentation to yield energy (Moore *et al.*, 2006). Other than that, some *Pseudomonas* species utilize NO_3 as final electron acceptor in nitrate respiration, a supplementary metabolic pathway. In Moore *et al.* (2006), *Pseudomonas chloritidismutans* utilized chlorate (ClO_3^-) as a substitute of energy generating electron acceptor. On the other hand, *Pseudomonas* spp. can use variety of organic compounds as the source of carbon and energy in order to survive in the environment that lacks nutrients (Igbinosa *et al.*, 2012).

2.3.5 Epidemiology

Pseudomonas is an opportunistic pathogen that causes infections in humans, animals and plants (Czekajlo-Kolodziej *et al.*, 2006; Franzetti & Scarpellini, 2007; Hirakawa *et al.*, 2010) due to ubiquitous lifestyle and nutritional diversity. *Pseudomonas* spp. can also be found in hospital, intensive care unit and can be isolated from hand creams, bar soaps, germicide solutions for cleaning, and antiseptic solutions (Bruun *et al.*, 1976).

2.3.6 *Pseudomonas aeruginosa*

Among all *Pseudomonas* spp., *P. aeruginosa* is the most common species to be studied for the pathogenicity. *P. aeruginosa* is an opportunistic pathogen which leads immunocompromised patients to diseases like neutropenia, hematologic cancers, pneumonia, and sickle cell anemia (Igbinosa *et al.*, 2012). On the other hand, *P. aeruginosa* also results in chronic pulmonary disease for patients with cystic fibrosis. According to Shigeki *et al.* (2011), *P. aeruginosa* can produce many virulence factors such as exotoxin and biofilm and causes the bacterium to be a virulent pathogen.

In the research carried out by Stehling *et al.* (2008, 2010), the pathogenesis of *P. aeruginosa* is due to the production of extracellular enzymes and these enzymes, or known as the exozymes include alkaline protease, elastase, exotoxin A, exoenzyme S and hemolysin. These exozymes are produced through clinical infection and involved in the development of animal infections since they will break down host membrane and lyse cell.

2.4 Biochemical Characterization of *P. aeruginosa*

In Ningthoujam and Shovarani (2008), morphological, biochemical, and physiological tests were performed in order to characterize *P. aeruginosa* strain DN1 degrading p-nitrophenol. These include visualization of morphology under light microscope, motility test by hanging drop method, catalase test, oxidase test using oxidase disc, casein and starch hydrolysis, citrate utilization, Indole, MR, VP, NR, gelatin liquefaction, urease production, and Ulrich milk peptonization. Pigment production was also determined using different media such as LB, *Pseudomonas* F medium, *Pseudomonas* P medium, and PIA. The results were shown in Table 2.1.

Table 2.1: Biochemical and physiological tests of the PNP degrading isolate *P. aeruginosa* DN1

Tests	Results
Catalase	+
Casein hydrolysis	+
Starch hydrolysis	-
MacConkey's agar	+(growth as nonfermenter)
EMB (Eosine Methylene Blue) agar	+
Citrate	+
Motility	+
Glucose fermentation semi solid medium	-
Lactose broth	-
Indole	-
MR	-
VP	-
Gelatin liquefaction	+(rapid liquefaction)
Urea broth	+
NR	+
Ulrich Milk broth	Deep red(peptonization)
Oxidase	+
Lipase	+(showing clear halo)

(Source: http://www.scialert.net/fulltext/?doi=jm.2008.345.351&org=10)

2.5 Antibiotic Resistance of *P. aeruginosa*

In recent years, the emergence of *P. aeruginosa* strains which were resistant to multidrug was observed worldwide. The ability of *P. aeruginosa* to resist to multiple antimicrobial agents made this bacterium an opportunistic pathogen. The bacterium does not affect

healthy human. In fact, the bacterium causes alternation to the host defense systems (Gales *et al.*, 2001).

Resistance of antipseudomonal β-lactams and new generation cephalosporins, monobactams, and carbapenems were well described. In 1991, *P. aeruginosa* that produced Metallo-Beta-Lactamase (MBL) was first reported from Japan (Manoharan *et al.*, 2010). The resistance to β-lactams is due to several mechanisms such as AmpC β-lactamase production, extended-spectrum β-lactamases, a barrier to diffuse at outer membrane, and efflux mechanisms. The formation of bacteria biofilm also contributed to the antibiotic resistance of *P. aeruginosa* (Gales *et al.*, 2001). These mechanisms limited therapeutic options for diseases.

According to the study conducted by Lister *et al.* (2009), *P. aeruginosa* exhibited highest resistant rates to fluoroquinolones like ciprofloxacin and levofloxacin. This trend was reported from patients in Intensive Care Units (ICUs). The patients in ICUs also have higher rate of resistance than hospitalized patients towards β-lactamase (Lister *et al.*, 2009).

3.0 Materials and Methods

3.1 Samples Collection

Samples were collected from rivers and aquaculture farms. Twelve samples collected were from the surface water of Permai River, Sarawak River Kiri (Bintawa industrial zone and Muara Tuang area), and Sarawak River Kanan (Siniawan and Waterfront area). Figure 3.1 shows Sarawak River Basin. Four water samples were collected Permai River while two samples were collected from each of the Waterfront, Muara Tuang, Bintawa, and Siniawan rivers. The water samples were taken using sterile falcon tubes and kept in icebox before transporting to laboratory for processing.

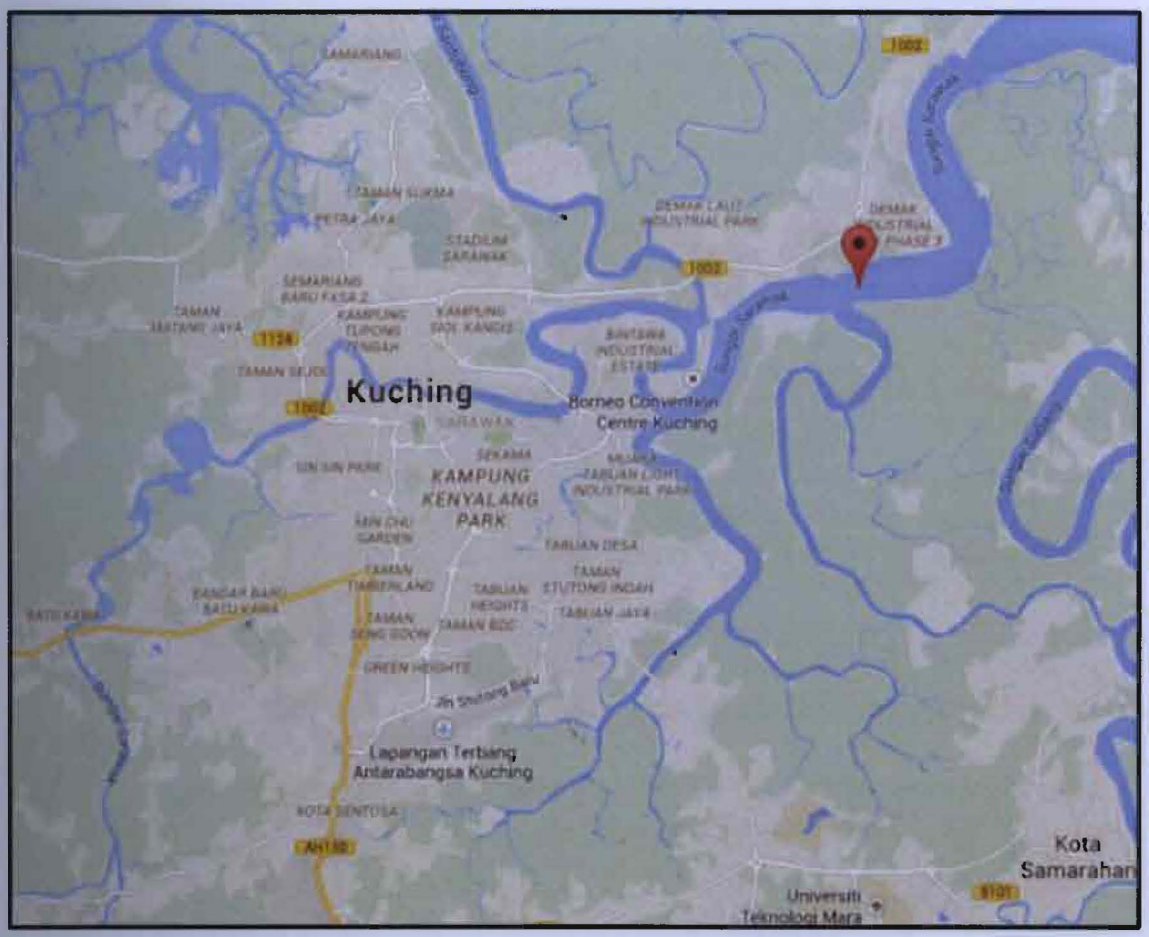

Figure 3.1: Sarawak River Basin. Sarawak River Basin has two major tributaries which are Sarawak River Kiri and Sarawak River Kanan.

Four aquaculture farms located at Sampadi, Bako 1, Bako 2, and Kampug Haji Baki were selected for samples collection. The samples collected from aquaculture farms include water, sediment, and cultured species. All water and sediment samples from Asia Aquaculture farm at Lundu (Appendix 1), Bako 1, Bako 2, and aquaculture farm, Kampung Haji Baki were taken using sterile falcon tubes while *Clarias gariepinus* or catfish (local name: *ikan keli*) was caught freshly using sterile polyethylene bags from Kampung Haji Baki. All samples were then kept in icebox containing ice and transported to laboratory within 24 hours for processing.

Eighteen water and sediment samples were collected from Asia Aquaculture farm located at Sampadi, Lundu. Twenty-seven water samples were collected from Asia Aquaculture farm at Bako, Kuching, sixteen water and sediment samples were collected from Bako 1 while eleven water and sediments samples were collected from Bako 2. For aquaculture farm at Kampung Haji Baki, thirteen water samples and a catfish were collected. All the samples were then labeled properly and processed immediately upon arrival in the laboratory.

3.2 Isolation of *P. aeruginosa*

Homogenization of water and sediment samples was performed by flicking the falcon tubes. Then, 50 µL of each water and sediment samples collected were taken directly from and spread onto CHROMagar™ *Pseudomonas* using spread plate technique. The surface, mouth, intestine (front, middle, end), and anus of catfish were swabbed using sterile cotton bud and spread onto CHROMagar™ *Pseudomonas* for isolation of *P. aeruginosa*. Meanwhile, the water and sediment samples, surface, mouth, intestine, and anus of catfish

were also spread plated onto TSA to obtain the total bacteria count of bacteria in the samples collected. After incubation at 37 °C for 24 hours, bacterial colonies formed were enumerated. The bacteria present in the samples were expressed as:

$$\text{Colony forming units (CFU)} = \frac{\text{Direct bacteria cell count x Dilution factor}}{\text{Volume plated (mL)}}$$

For the isolation of *P. aeruginosa*, one or two single colonies from each CHROMagar™ *Pseudomonas* plate were randomly selected before sub-culturing onto TSA agar slant for further study and storage purposes. The isolated *P. aeruginosa* were further characterized using conventional biochemical tests such as Gram staining, MacConkey agar test, oxidase test, Triple Sugar Iron (TSI) test, citrate utilization test, catalase test, starch hydrolysis test, and nitrate reduction test (refer Appendix 3).

3.3 Cultural Characterization of *P. aeruginosa*

Gram staining method was carried out according to Cappuccino and Sherman (2004). A drop of distilled water was placed on the center of slide before a small amount of bacterial colonies were placed onto the slide. The distilled water was then spread into a thin area and heat fixation was performed.

The heat-fixed smear of cell was flooded with crystal violet for 1 minute and then washed gently with tap water. The smear was flooded with Gram's iodine mordant for a minute before gently washing procedure. Next, the smear was decolorized with 95% ethanol until the ethanol runs almost clear. Then, the smear was washed gently using tap water. Lastly, the smear was counterstained with safranin for 45 seconds and washed gently with tap water.